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# Author's Accepted Manuscript

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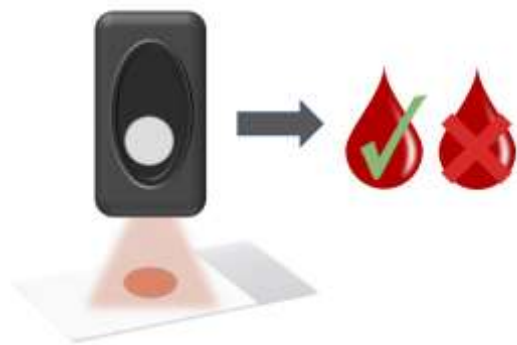
# Feasibility of a Handheld Near Infrared Device for the Qualitative Analysis of Bloodstains

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## Abstract

One of the most common tasks in criminal investigation is to determine from which tissue source a biological fluid stain originates. As a result, there are many tests that are frequently used to determine if a stain is blood, semen or saliva by exploiting the properties of certain molecules present within the fluids themselves. These include chemical reagents such as the Kastle-Meyer or Acid Phosphatase tests, as well as other techniques like the use of alternative light sources. However, most of the tests currently available have some major drawbacks. In this study, a handheld near-infrared spectrometer is investigated for the specific identification of deposited bloodstains. First, a calibration was carried out by scanning over 500 positive (blood present) and negative (blood absent) samples to train several predictive models based on machine learning principles. These models were then tested on over 100 new positive and negative samples to evaluate their performance. All models tested were able to correctly classify deposited stains as blood in at least 81% of tested samples, with some models allowing for even higher classification accuracy at over 94%. This suggests that handheld near infrared devices could offer great opportunity for the rapid, low cost and non-destructive screening of body fluids at scenes of crime.

**Graphical Abstract**

**Keywords:** Near Infrared; Spectroscopy; Body Fluids; Blood; Forensic; SCiO.

**1. Introduction**

According to the Office for National Statistics, over 1.1 million violent offences occurred within the UK in 2016 [1]. Forensic investigation of these types of offences is likely to involve the identification and analysis of stains from biological fluids, such as blood, semen or saliva, which may be present on a variety of surfaces (both porous and non-porous). The presence or absence of these stains can have a significant effect on the outcome of an investigation, both by elucidating the sequence of events involved in the offence and by linking individuals to an offence via the analysis of DNA. The availability of quick, inexpensive, facile and non-destructive tests that can be carried out at the scene of crime is thus of crucial importance for the timely and accurate identification of body fluids.

Currently, there is a wide range of 'in-field' techniques that help forensic investigators determine the identity of crime scene stains. However, most of these techniques are presumptive and may only be used to indicate what the possible identity of a stain may be. A confirmatory test is often required to avoid the risk of false positive or negative results [2]. One well-known example of a presumptive technique is the use of alternate light sources (ALS) to enhance body fluid stains. This technique is particularly useful in visualising stains invisible to the naked eye (or those deposited on dark surfaces) and is based on the fluorescence emission of certain molecules within these fluids after excitation at a particular wavelength [3, 4]. Although fast and simple, this

technique is largely non-specific, as many and background substrates can fluoresce at the same wavelength [2]. In addition, blood does not fluoresce upon light source illumination and is therefore incompatible with ALS testing [3, 4]. Another disadvantage of this technique is that certain types of radiation (i.e. ultra-violet) may cause damage to DNA present within the sample [5, 6].

Other presumptive tests for body fluids include various chemical reagents, such as the Kastle-Meyer (KM) test or luminol for blood [7-10], the acid phosphatase (AP) test for semen [11, 12], or the Phadebas® test for saliva. Most of these are based on specific chemical properties of molecules present in the target body fluid, such as the peroxidase-like activity of haemoglobin (KM test), or the action of salivary amylase on starch (the Phadebas® test) [13, 14]. Such activity is then used to generate a change of colour or luminescence (as in the case of luminol) to indicate a positive result. While in some cases a surface stain will be analysed by first rubbing a piece of filter paper on it and then applying the chosen reagent to the paper, when the amount of sample is limited the reagent will be applied directly to the stain on its original surface. This in turn may prevent the stain from being used for subsequent DNA testing. In addition, some chemical presumptive tests require total darkness for proper visualisation (i.e. Luminol) [9, 10] and most of such reagents may only identify one type of body fluid per test.

More recently, body fluid identification has been performed using immunological chromatographic testing cartridges, which exploit specific antibody-antigen interactions to differentiate between different fluids. One commercially available example of this technology is the Hematrace® device used for the identification of bloodstains [15]. Although quick and specific, the main disadvantage of such devices is that they are sample destructive (i.e. fluid samples cannot be recovered from the cartridge after testing), an important issue in situations where the amount of sample available is small.

In view of these challenges, it has become necessary to develop new techniques that can be used to identify biological fluid stains directly at the scene of a crime. Recent technological progress has allowed manufacturers to miniaturize a number of analytical instrumentation, resulting in the production of portable and handheld devices that enable scientists to carry out analysis at any location [16-20]. The development of devices for the examination of evidence at crime scenes, without the need for transportation to a specialist laboratory (which is often costly and time-consuming) is therefore of great interest to the forensic body fluid identification community.

Current efforts to achieve this goal have largely focused on spectroscopic techniques, widely used in many other analytical disciplines [21, 22]. A number of studies have already been performed to determine the effectiveness of various spectroscopic techniques for the identification of biological fluids, including ultraviolet-visible (UV-vis) [21], infrared (IR) [23-25] and Raman spectroscopy [26-29]. While UV radiation may damage DNA contained within a fluid sample [5, 6], IR spectroscopy has proven to be an interesting means to 'fingerprint' body fluids as this technique can determine the presence of specific classes of molecules in body fluids with sample volumes as small as 10 - 20  $\mu$ L [23-25]. Nevertheless, most of the IR studies carried out so far employ benchtop instruments that cannot be taken to the crime scene. There is also the additional disadvantage that the presence of any water in the sample could interfere with the detection of certain types of analytes [25]. There have been successful attempts to identify the signature of different body fluids using Raman spectroscopy, coupled with near-infrared (NIR) excitation [26-28]. However, when not coupled to NIR, Raman spectroscopy presents some drawbacks, such as the strong fluorescence generated by some substrates (such as glass), which can mask signals produced from body fluid samples [26, 27].

Within the context of forensic analysis, NIR spectroscopy can offer several advantages including:

- Little or no sample preparation, which may allow users to analyse evidence *in situ* i.e. at the scene of crime [30].
- Rapid sample scanning time (completed within a few seconds), which could significantly reduce the amount of time spent on a case [30].
- Non-destructive analysis, preventing the loss of samples needed for downstream DNA processing [30].
- Ability to scan samples inside glass or plastic containers, which may be potentially useful in applications such as the analysis of seized drugs of abuse, or the analysis of evidence at the scene of crime without unwrapping it or taking it out of its container [31-34].
- Minimal masking of analytes bands due to the presence of water molecules are well known and localised [35, 36].
- No issues with fluorescence from background substrates, which could potentially mask the presence of biological samples [26-28].

This study therefore investigates the use of a handheld NIR device, the SCiO® made by Consumer Physics®, for the identification of body fluid stains. SCiO® is a handheld NIR spectrometer, able to scan solid and liquid samples in the range of 700-1100 nm, the region of the third overtone in the NIR spectrum [30]. The detector of this device measures attenuated reflectance from the sample scanned, which means the device allows the user to scan samples on opaque surfaces. The device is connected to a mobile phone by Bluetooth and controlled through the mobile app SCiO® Lab. Through this app the device sends the data obtained from the sample and displays it in a user-friendly format while, at the same time, uploads the data to an online 'cloud' database so it can be studied on any other device.

As blood is one of the body fluids most commonly found at the scene of crime, bloodstains were selected as the focus of this investigation. First, a number of classification models were created using online software tools provided by Consumer Physics® (<https://www.consumerphysics.com/>, Israel) after scanning samples of blood deposited on different surfaces. Although full details are proprietary, these tools utilise a "powerful cloud-based machine learning" method which is based on Partial Least Square Regression (PLSR), a multivariate often used as an alternative to Principal Component Analysis (PCA) [37]. After data processing, the performance of each model was tested by using them to identify new samples on glass. Only a few studies have investigated the use of NIR spectroscopy for the characterization of biological evidence in a forensic context [38, 39]. Edelman *et al.* [38] showed that NIR spectroscopy has the potential to be employed not only for the identification of body fluids but also to estimate the time since deposition of a stain. The study however employed a benchtop instrument.

Pereira *et al.* [38] recently sought to identify bloodstains for forensic purposes utilising the MicroNIR™ handheld NIR spectrometer produced by US company Viavi. Whilst successfully able to differentiate human blood from several animal blood and non-blood samples, this specific device is unlikely to be amenable to current crime scene testing processes. First, it may be challenging for scenes of crime officers and police responders (who are likely to have limited spectroscopy training) to operate the specialized chemometric software required for data analysis. Conversely, SCiO® Lab software allows the development of dedicated applications for the testing of specific sample types, which allow personnel with no background in NIR analysis to use the device "as is" with minimal user input. Second, unlike the SCiO® device which is currently priced at

around £225, the MicroNIR™ may cost between £16-20,000 depending upon application. Purchasing an array of such devices for crime scene deployment is likely to be unfavourable with forensic service providers in a time where analysis budgets are increasingly restricted. Lastly, the MicroNIR™ device does not support wireless operation of 'cloud'-based data storage.

All this considered, the success of this study would mean that a handheld device might have the potential to be used as a fluid identification technique directly at the scene of crime with no need of sample preparation, thus, reducing the cost and time needed for forensic examinations.

## 2. Materials and Methods

### 2.1. Device and software

All scans were carried out using the SCiO® NIR scanner from Consumer Physics (Tel Aviv, Israel) as illustrated in Figure 1. Samples were scanned on a flat benchtop, with the device placed over the sample using the shade provided in order to protect the sample from any external source of light. All samples on glass slides or thin surface materials were kept at a height of 5 cm, preventing the device from inadvertently scanning any surface below the samples. Data collection was controlled via the mobile app SCiO Lab® for Android.

### FIGURE 1 HERE

**Fig. 1.** Representation of the workflow used with the SCiO® NIR scanner for the analysis of bloodstains.

### 2.2. Instrument calibration

#### 2.2.1. *Blood on glass slides*

Prior to the deposition of samples, all slides were cleaned with ethanol (Sigma-Aldrich, Dorset, UK). Blood samples were taken by fingerstick using safety Lancets (Sarstead, Numbrecht, Germany) after informed consent was obtained from 20 healthy donors (4 males and 16 females) From each donor, five separate samples (n=100) were taken by letting blood droplets fall onto glass slides within an area of approximately 1x1 cm. After drying for two hours at room temperature [38], each sample was scanned three times. A total of 300 scans were performed [24].



### 2.2.2. *Blood on surfaces*

Aside from glass, six additional surfaces on which blood is likely to be deposited at the scene of a crime (i.e. tile, wood, 100% cotton, 100% leather, 100% acrylic and 100% cotton) were also prepared and divided into individual 6x6 cm areas. In a similar manner to the samples on glass slides, five blood samples from 8 of the original 20 donors (n=40) were deposited within a central area (~ 1x1 cm) of each surface type. Each stain was scanned three times to give 120 total scans per surface type.

### 2.2.3. *Blood-like substances*

Along with blood samples, eight other substances that resemble fresh or dry blood (i.e. red wine, red ink, tomato sauce, fake blood, coffee, red food colouring, red paint and beet root juice) were also deposited on glass slides to test for potential false positive results. Each substance was deposited in 100  $\mu$ L volumes (to represent that of an average blood droplet [40]) in 8 sets of 5 samples (n=40) and let dry at room temperature for two hours. Each sample was scanned three times to give 120 total scans.

## 2.3. Chemometric model construction

Data processing and chemometric model construction was carried out using online software tools provided by Consumer Physics® specifically for the device. The software uses PLSR for model construction with Mean-Centering applied by default. Several pre-processing methods were first investigated, including Mean-Centering, Standard Normal Variate (SNV) and First and Second Derivative [41-44]. This was carried out through a cross-validation process to identify the pre-processing methods that resulted in the most accurate classification. Based on the results obtained from the cross-validation study Mean-Centering and SNV were chosen (Figure S1). In addition to the full spectra, a specific wavelength range (800-940 nm) was also investigated and an outlier detection function was applied to all the models.

Twelve models were created of which: four models included all the datasets (sections 2.2.1, 2.2.2 and 2.2.3); four included data from blood on glass slides (section 2.2.1) and four included data from blood on different surfaces (section 2.2.2).

## 2.4. Validation of the models

In order to evaluate the performance of the created models when applied to new samples, a 'test' validation was undertaken by scanning blood samples from 10 new donors ( $n=50$ ) that were not included in the model construction stage [42, 45, 46]. These samples were prepared in the same way as described in section 2.2.1. In addition, new samples of the 8 blood-like substances previously described ( $n=5$  for each substance) were prepared as described in section 2.2.3. These, together with 10 clean glass slides, were used as negative controls, making a total of 50 negative controls. All samples were scanned once.

Parameters specific to qualitative methods based on binary response were used to assess model performance, as only two outputs (i.e. 'blood' or 'not blood') were possible. These parameters included *false positive rate* ( $FP/(TP+FN)$ ), *false negative rate* ( $FN/(TN+FP)$ ), *sensitivity* ( $TP/(TP+FN)$ ), *specificity* ( $TN/(TN+FP)$ ) and *likelihood ratio for a positive result (LR+)* ( $(1 - \text{False negative rate})/\text{False positive rate}$ ) [47-50].

Once these parameters were calculated, all models were plotted on a receiver operating characteristic (ROC) curve. The ROC curve is a common graphical method of comparing the performance of two or more diagnostic models as a function of Sensitivity against 1-Specificity (false positive rate) [51]. The closer a model is to the upper-left corner of the curve, the more accurate it is likely to be.

### 3. Results and discussion

#### 3.1 Data Collection

First (for baseline offset differences removal) and Second Derivative (for baseline offset and slope differences removal) and SNV (as a scatter-corrective pre-processing) were initially investigated as potential pre-processing methods [41, 43, 44]. Furthermore, NIR spectra can display peaks that are characteristic of water molecules; within the range scanned by the SCiO device there are two water bands, at 760 nm and 970 nm [36]. Therefore, in addition to using the data from the full spectrum (i.e. 700-1100 nm), a selected wavelength range (i.e. 800 and 940 nm) was also used to avoid potential variability due to different amounts of water present in the samples. Such wavelength range would still retain the band for a characteristic component of blood, oxyhaemoglobin, at 930 nm [52]. Models were created using either the full spectrum data or the selected wavelength data. All the models additionally included an 'outlier detection' function, forcing the device to give

a null result if it was not able to match the spectrum from a new sample with the spectra from the calibration data collection. Lastly, models were created with these different forms of pre-processing using data from particular subsets of bloodstain samples. This included all scanned samples, blood samples on glass slides and blood on all the surfaces except glass (to see how well the model would perform without information regarding the surface). The entire experimental workflow, including all sample subsets and pre-processing methods used to create each model, is outlined in Figure 2.

#### FIGURE 2 HERE

**Fig. 2.** Flowchart summarizing the different stages of this study. Raw = Mean-centered raw data. SNV = Standard Normal Variate. SW = Selected Wavelength range (800 – 940 nm).

In the cross-validation study, First and Second Derivative methods did not demonstrate any substantial improvement in the prediction values compared to mean-centered raw data and SNV. More specifically, while correct classification for the ‘true positives’ was comparable amongst all the methods (97%), for the ‘true negatives’ a decrease was observed with 1st and 2nd Derivative (75% and 83% respectively versus 85% and 87% for mean-centering and SNV). Same results were obtained with both the full spectra and the selected wavelength range. These models were therefore tested to evaluate their performance against test samples.

#### 3.2. Model validation

Once diagnostic models are created, it is essential to carry out a validation to evaluate their performance with new samples [45]. There are two ways this can be achieved: cross-validation or ‘test’ validation [42, 46]. Leave-One-Out Cross-Validation (LOOCV) is a process carried out by the software used for model creation, where the program takes out one sample at a time from the calibration set of samples, tests the model on this sample and repeats the process again for every sample until all samples have been tested. This is common in studies where the number of available samples is small and limited. On the other hand, ‘test’ validation is a process where the samples available are split into two sets, a calibration set and a test set, with the former consisting of 60-70% of the entire sample pool [45]. Since a sufficient number of samples were available for this study ( $n > 50$ ) [45], a ‘test’ validation was carried out to evaluate model performance. This was done by scanning 50 positive controls

and 50 negative controls with the device using the testing mode, which allows the user to scan a new sample and analyse it with the model(s) created. At the end of the analysis the most likely classification for the sample, along with confidence level of said classification, is provided by the software (Table S1). Table 1 shows the number of true and false positives and negatives as well as the values of false positive rate, false negative rate, sensitivity, specificity and likelihood ratio for all the models.

#### TABLE 1 HERE

Once the parameters were calculated the values of Sensitivity and 1-Specificity (false positive rate) of all the models were represented in a ROC space (Fig. 3.). In this type of representation, the models that are closer to the upper left corner of the graph (i.e. high sensitivity and low false positive rate) would be considered as those with the best performance.

#### FIGURE 3 HERE

**Fig. 3.** ROC space (Sensitivity against 1-Specificity ) representing the performance of 12 models (i.e. A – L).

These results show the potential of some of the models used in this study such as models A, B or C with probabilities of correct classification of positive samples in 94%, 90% and 86% of test cases, and negative samples in 86%, 84% and 82% of the test cases respectively. An LR+ of 6.71 was obtained in the case of model A (mean-centered raw data from all samples), which can be considered as a model with a “very good” qualitative strength [53].

These metrics indicate that the SCiO® device could potentially be used as a screening tool for a fast, non-destructive identification of blood and, pending further study, of other body fluids. However, the interpretation of these reliability measures depends on the context and purpose of the measurement. These studies would include a further characterization of blood [38, 52] and other body fluids within the region scanned by this device, as it is a region not commonly used due to its lower resolution and intensity of the peaks, and using other available software to build chemometric models based on multivariate analysis. The latter might include principal component analysis (PCA) [54, 55], or partial least squares (PLS) [54], both of which would take into

account data from the entire spectral range, thus allowing the identification of different body fluids that may share common molecules in their composition.

#### **4. Conclusions**

The goal of this study was to determine if the SCiO<sup>®</sup> NIR device may be able to differentiate bloodstains from blood-like substances that may also be present at the scene of a crime. The results showed that it was possible to create a model with which the device was able to identify blood correctly in 94 % of the cases with a rate of false positives of 14 % of the blood-like samples. This means that SCiO<sup>®</sup> has the potential to become a rapid (with analysis taking less than a minute per sample) and non-destructive method for the screening of body fluids, which would permit crime scene analysis without any sample preparation, reducing the time and cost associated with the analysis of this evidence. Furthermore, its user-friendly interface would allow crime scene officers to make use of this device without the need of spectroscopy experts to be present, thus reducing the risk of cross contamination due to the presence of too many individuals.

The study reported herein evaluated the feasibility of this method for blood stain identification; it must be noted that further development and validation within real life scenarios representative of the usual framework in forensic sciences is required to determine if this device is fit to be used at a real crime scene. In addition to this, the authors would like to suggest to further study the effect of a wider range of substrates as well as the potential use of this device for the estimation of the age of the sample. Nevertheless, once these aspects have been studied and the method is properly validated, it is expected that this device will prove useful and will be widely adopted in crime scene investigation.

#### **5. Acknowledgements**

We would like to gratefully acknowledge all the donors that selflessly provided samples for this study. Additional thanks are given to Dr. Arundhuti Sen for her valuable input and advice.

**Table 1.** Summary of the results from model validation. Model A = mean-centered raw spectral data from all samples, Model B = SNV applied to all samples, Model C = mean-centered raw spectral data from all samples + selected wavelength range 800 - 940 nm, Model D = All samples applying SNV on the range 800 - 940 nm, Model E = mean-centered raw spectral data from blood samples on glass, Model F = SNV applied to blood samples on glass, Model G = mean-centered raw spectral data from blood samples on glass + selected wavelength range 800 - 940 nm, Model H = blood samples on glass applying SNV on the range 800 - 940 nm, Model I = mean-centered raw spectral data from blood samples on different surfaces, Model J = SNV applied to blood samples on different surfaces, Model K = mean-centered raw spectral data from blood samples on different surfaces + selected wavelength range 800 - 940 nm, Model L = blood samples on different surfaces applying SNV on the range 800 - 940 nm.

	A	B	C	D	E	F	G	H	I	J	K	L
<b>False Positive Rate</b>	0.14	0.16	0.18	0.16	0.32	0.22	0.30	0.16	0.52	0.32	0.88	0.40
<b>False Negative Rate</b>	0.06	0.10	0.14	0.20	0.20	0.20	0.14	0.30	0.38	0.46	0.04	0.18
<b>Sensitivity</b>	0.94	0.90	0.86	0.80	0.80	0.80	0.86	0.70	0.62	0.54	0.96	0.82
<b>Specificity</b>	0.86	0.84	0.82	0.84	0.68	0.78	0.70	0.84	0.48	0.68	0.12	0.60
<b>Likelihood Ratio</b>	6.71	5.63	4.78	5.00	2.50	3.64	2.87	4.38	1.19	1.69	1.09	2.05

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### Highlights:

- A handheld Near Infrared spectrometer was used for the identification of blood stains
- Twelve chemometric models were constructed
- The prediction performance was evaluated on 100 samples
- The models show low false positive and false negative rate.



